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Landscape of somatic mutations in sporadic GH-secreting pituitary adenomas

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Short title: genetic pattern of GH-secreting adenomas

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Abstract

Context: Alterations in the cAMP signalling pathway are common in hormonally-active endocrine tumors. Somatic mutations at *GNAS* are causative in 30-40% of GH-secreting adenomas. Recently, mutations affecting the *USP8* and *PRKACA* gene have been reported in ACTH-secreting pituitary adenomas and cortisol-secreting adrenocortical adenomas, respectively. However, the pathogenesis of many GH-secreting adenomas remains unclear.

Aim: Comprehensive genetic characterization of sporadic GH-secreting adenomas and identification of new driver mutations.

Design: Screening for somatic mutations was performed in 67 GH-secreting adenomas by targeted sequencing for *GNAS*, *PRKACA*, and *USP8* mutations (n=31) and next-generation exome-sequencing (n=36).

Results: By targeted sequencing known activating mutations in *GNAS* were detected in 5 cases (16.1%), while no somatic mutations were observed in both *PRKACA* and *USP8*. Whole exome sequencing identified 132 protein-altering somatic mutations in 31/36 tumors with a median of 3 mutations per sample (range: 1-13). The only recurrent mutations have been observed in *GNAS* (31.4% of cases). However, 7 genes involved in cAMP signalling pathway were affected in 14 of 36 samples and 8 samples harbored variants in genes involved in the calcium signalling or metabolism. At the enrichment analysis, several altered genes resulted to be associated with developmental processes. No correlation between genetic alterations and the clinical data was observed.

Conclusions: This study provides a comprehensive analysis of somatic mutations in a large series of GH-secreting adenomas. No novel recurrent genetic alterations have been observed, but the data suggest that beside cAMP pathway calcium signalling might be involved in the pathogenesis of these tumors.

Introduction

Pituitary tumors represent approximately 15% of all primary intracranial lesions. Growth hormone (GH)-secreting pituitary adenomas are the second most frequent type of hormone-producing pituitary tumors, after prolactin-secreting adenomas¹. Excessive secretion of GH causes gigantism during childhood and acromegaly in adults, with significant morbidity due to clinical complications involving cardiovascular, respiratory, and metabolic systems.^{2, 3}

The monoclonal origin of most pituitary adenomas indicates that these tumors derive from the replication of a single cell that acquired growth advantage. The latter has been suggested to result from genetic or epigenetic alterations leading to activation of proto-oncogenes or inactivation of tumor suppressor genes^{4, 5}. However, despite intensive investigations, little is known about the genetic causes of pituitary adenomas. The only mutations identified to date in a significant proportion (30-40%) of sporadic GH-secreting adenomas occur in the gene encoding the α subunit of the stimulatory G protein (*GNAS*).⁶⁻⁹ These somatic activating mutations (*gsp* mutations), found in codon 201 and 227, prevent hydrolysis of GTP, leading to a constitutive activation of the cAMP pathway, which in somatotrophs and in other endocrine cells acts as a mitogenic signal^{10, 11}. In somatotrophs the *GNAS* transcript is expressed mainly from the maternal allele, due to tissue-specific paternal imprinting^{12, 13}. Consistently, *gsp* mutations in sporadic GH-secreting adenomas are found on the maternal allele¹⁴ and partial loss of this imprinting is present in tumors negative for *gsp* mutations¹⁵, further supporting the involvement of *GNAS* locus in pituitary tumorigenesis. So far, the screening for mutations in other G-protein subunits in pituitary tumors has given negative results.¹⁶⁻¹⁸

Genetic alterations in other genes involved in cAMP signalling have been identified as cause of other endocrine tumors. A reduced expression and/or function of the PKA regulatory subunit type I α (*PRKAR1A*) due to loss-of-function mutations, leading to an abnormal cAMP pathway activation, causes GH-secreting pituitary adenomas in Carney complex, an autosomal dominant familial syndrome^{19, 20}. To date, mutations of *PRKAR1A* gene have been rarely found in sporadic pituitary tumors^{21, 22}, although a reduced *PRKAR1A* expression resulting from increased proteosomal degradation has been described in sporadic GH-secreting tumors¹⁰. Reduced cAMP degradation caused by mutations in *PDE11A* and *PDE8B*, coding for members of the phosphodiesterase (PDE)

family, have been involved in adrenocortical hyperplasia, adenomas and cancer as well as in testicular germ cell tumors^{23, 24}. However, genetic variants of *PDE11A4* contribute only marginally to the development of GH-secreting adenomas²⁵. Recently, mutations affecting the gene encoding the catalytic subunit α of the PKA (*PRKACA*) have been reported in a large proportion of cortisol-secreting adrenocortical adenomas²⁶⁻³⁰, resulting in increased PKA activity³¹. Nevertheless, no hot spot mutations of *PRKACA* have been identified in a large cohort of GH-secreting adenomas³². Finally, a recurrent somatic mutation in the *GPR101* gene, which encodes an orphan G-protein-coupled receptor, has been recently reported in some adults with acromegaly (4% of cases).³³

In addition, epidermal growth factor receptor (EGFR) overexpression has been described in hormonally active pituitary adenomas³⁴ and role for EGF and its receptor in the development and/or progression of pituitary tumors has been hypothesized³⁵. Dominant mutations in the deubiquitinase *USP8* gene that promote activation of EGFR signalling have been also found in ACTH-secreting pituitary adenomas by exome sequencing³⁶. Finally, germline mutations of genes such as the aryl hydrocarbon receptor interacting protein (*AIP*), the menin (*MEN1*) and the p27 (*CDKN1B*) have been reported in genetic syndromes associated with acromegaly (i.e. familial isolated pituitary adenoma and multiple endocrine neoplasia type 1 and 4) and in a low percentage of young acromegalic patients.³⁷

Recently, Valimäki et al. investigated a small group of 12 patients with GH-secreting adenomas by whole-genome sequencing and SNP array and did not find any novel recurrent genetic alteration.³⁸ Aim of the present study was to perform a comprehensive genetic characterization of a large series of GH-secreting adenomas to identify novel genetic alterations potentially involved in tumorigenesis and/or in clinical outcome. To this aim, we used both targeted direct sequencing of *GNAS*, *PRKACA* and *USP8* genes and next-generation exome sequencing.

Material and methods

Tissue samples, patients and clinical annotations

Sporadic GH-secreting adenomas without familial or syndromic presentation were recruited in the present study. Accordingly, a total of 81 fresh frozen tumors were collected from 4 different participating European centers. If available, corresponding peripheral blood was also collected for the

analysis. Inclusion criteria for participating in the study were a certified histological diagnosis of benign GH-secreting adenomas and available clinical data. The DNA was isolated as described previously.³⁹ Qualitative and quantitative evaluation of the DNA was assessed by electrophoresis in a 1% agarose gel and spectro-photometrically at 260 nm, respectively. At the first screening, 14 tumor samples have been excluded due to insufficient DNA quality, so that the final series included a total of 67 GH-secreting adenomas. Among them, the tumor samples were subdivided into two groups according to the availability of corresponding leukocyte-DNA essential for next generation whole exome-sequencing. Thus, 31 tumor samples underwent targeted direct sequencing for the analysis of selected genes (*GNAS*, *PRKACA* and *USP8*) (Group 1), while the remaining 36 cases with corresponding leukocyte-DNA were investigated by whole exome-sequencing (Group 2).

Clinical parameters, such as sex, age at diagnosis, date of surgery, tumor size, GH and IGF-I levels, presence of acromegaly-related complications, as well as follow-up data were collected for all patients at the local centers. All the patients gave written informed consent and the study was approved by the ethics committee at each participating institution.

Targeted and whole-exome sequencing and data analysis

For the targeted sequencing analysis we focused on gene domains harboring alterations known or supposed biologically relevant in endocrine active tumors, i.e. known gain-of-function *GNAS* mutations (codon 201 and 227), mutations in the catalytic domain of the *PRKACA* (exon 7 and 8) and in the 14-3-3- binding domain and the MIT (Microtubule Interacting and Transport)-domain (exons 1, 2, 3) of the *USP8*, which is reported to be involved in regulating *USP8* catalytic function. In addition, we also evaluated the presence of the known hot spot *GNAS* mutations. The primers used for the targeted direct sequencing were generated with the Program Primer3Plus³⁹.

The complete list of the primers is reported in the ***Supplementary Table 1***. In brief, PCR was performed on 1 µl of diluted DNA (2 ng/µl) in a final volume of 25 µl containing 1,5 mM MgCl₂, 0,2 µM of each primer, 200 µM dNTPs and 1 U *Taq* DNA Polymerase. The reaction was started with an initial 95 °C denaturation step for 3 min, followed by 30 cycles of denaturation at 93 °C (20 sec), annealing at 58 °C (30 sec) and elongation at 72 °C (1 min). Direct sequencing of PCR products was

performed using the QuickStart Cycle Sequencing Kit (ABSciex) on a CEQ8000 DNA Analyzer (ABSciex).

For the next generation sequencing, exomes were enriched in solution and indexed with the use of the SureSelect XT Human All Exon 50Mb kit, version 5 (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed as paired-end reads of 100bp on a HiSeq2500 systems (Illumina, S. Diego, CA, USA) generating 8-14 Gb of sequence and an average depth of coverage between 110x and 170x on target regions. More than 95% of the target regions were covered 20 times or more. Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and base calling were performed with the use of Real-Time Analysis software (Illumina). Reads were aligned against the human assembly hg19 (GRCh37) using Burrows-Wheeler Aligner (BWA v 0.7.5a). Variant detection was done as described earlier.²⁶

Somatic variants have been evaluated by Polymorphism Phenotyping v2 tool (PolyPhen-2)⁴⁰ and SIFT algorithm (<http://sift.jcvi.org/index.html>)⁴¹. An unsupervised complete linkage clustering including the most relevant somatic mutations was performed by the Hamming distance as a similarity metric. The Gene Set Enrichment Analysis (GSEA) software was used for the gene enrichment and the functional annotation (MSigDB database v5.0)⁴². A canonical pathway analysis (1330 gene set) and a gene family analysis were also performed with the same software.

Statistical analysis

Median, interquartile range (IQR), and frequency were used as descriptive statistics. IGF-I values were expressed as percentage of the upper limit of the normal range (%ULN). The Fisher's exact test or the Chi-square test were used to investigate dichotomic variables, while a two-sided *t* test (or non-parametric test) was used to test continuous variables. A non-parametric Kruskal-Wallis test, followed by the Bonferroni *post-hoc* test, was used for multiple comparisons among several groups for non-normal distributed variables. Correlations and 95% confidence intervals (95%CI) between the total number of mutations and different clinical parameters were evaluated by linear regression analysis. Statistical analyses were performed using the GraphPad Prism (version 5.0, La Jolla, CA, USA) and

SPSS (PASW Version 21.0, SPSS Inc., Chicago, IL, USA) software. P values <0.05 were considered as statistically significant.

Results

Targeted DNA sequencing (Group 1)

A total of 31 patients affected by GH-secreting adenomas were included in this group. Minimum clinical data were available for 17 of them (10M&7F; median age: 46 yrs, range: 19-64; 16 macroadenomas and 1 microadenoma; median basal GH levels: 24.3 ng/mL, range: 2.3-333; median IGFI %ULN: 391, range: 266-590).

We observed the presence of known activating *GNAS* mutations in 5 out of 31 evaluated samples (16.1% of cases), i.e. a p.Arg201Cys substitution in 4 samples and a p.Gln227Leu in 1 sample. We did not identify any mutation in all the evaluated exons of *PRKACA* and *USP8* (**Table 1**). However, we detected different polymorphisms in the *USP8* gene: exon 1 (rs3131575 T/G heterozygous in 8 cases and homozygous in 1 case, rs11632697 G/C heterozygous in 14 cases and homozygous in 1 case, rs11632708 C/T heterozygous in 13 cases and homozygous in 1 case) and 14-3-3 binding domain (rs11638390 A/G heterozygous p.T739A in 14 cases and homozygous in 1 case) (**Table 1**). Allele frequencies did not differ significantly from frequencies reported in dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) (**Supplementary Table 2**).

Next-generation exome-sequencing (Group 2)

The histopathological and clinical parameters for the patients included in this analysis are reported in the **Table 2**. At the whole-exome sequencing, we identified a total of 132 protein-altering somatic mutations in 36 samples, resulting in a median of 3 somatic mutations in exonic regions per sample (range: 0-13). The genetic alterations included 109 missense and 7 nonsense mutations, 12 frameshift, 2 direct splicing and 2 indel variations. According to the PolyPhen-2, 39 mutations were classified as probably damaging, 25 as possibly damaging, and 41 as benign. The entire list of the somatic

mutations including localization, gene symbols and transcripts is reported in the *Supplementary Table 3*.

We identified a subgroup of patients without any mutation (negative, n=5), a subgroup with a low number of mutations (n 1-3, n=14) and a subgroup with a high number of mutations (n >3, n=17) (**Figure 1**). No significant correlation was observed between the total number of mutations and the evaluated clinical data, such as sex, age, tumor size and extension, and the initial GH levels.

The most frequent genetic alterations were the known gain-of-function mutations in the *GNAS* gene. Specifically, they were detected in 11 cases (31.4% of total, 10 of them being females), encoding p.Arg201Cys substitution in 7 samples, p.Arg201His in 2 samples and p.Gln227Leu in 2 samples. No difference was observed in total number of mutations between the tumors with or without *GNAS* mutations (**Figure 1**). No further genetic alterations were found in more than one sample in this series. Even comparing the list of the mutated genes with that of a recent paper on whole-genome sequencing in 12 GH-secreting adenomas³⁷, no additional recurrent somatic genetic alterations were observed.

However, some non-recurrent heterozygous somatic variants were observed in genes encoding G-protein coupled receptors (GPCR), such as the chemokine receptor 10 (*CCR10*) and the olfactory receptor *OR51B4*, which are coupled to the Gs protein (activation of the cAMP signalling pathway), and the M3 muscarinic cholinergic receptor (*CHRM3*), which functions through Gq (activation of the inositol trisphosphate/calcium signalling pathway). Moreover, other non-recurrent alterations were found in genes coding for proteins involved in cAMP signalling pathway other than *GNAS*, such as the $\alpha 2$ catalytic subunit of the AMP-activated protein kinase (*PRKAA2*), the G-protein-coupled receptor kinase 3 (*GRK3*, alias *ADRBK2*), the A1 subunit of the lysosomal H⁺ ATPase (*ATP6V0A1*). Taken together, the mutations in genes involved in the cAMP signalling affected 14/36 samples (38.9% of total). Among them, 9 samples presented only *GNAS* mutations, 2 samples mutations at *GNAS* and other genes of the cAMP signalling and 3 only mutations in other genes encoding GPCR or other members of the cAMP signalling. The corresponding details are reported in the **Table 3**.

Finally, a number of altered genes associated at different levels with the Ca²⁺ signalling and metabolism (i.e. involving both extra- and intracellular compartment) were observed in 8 cases (22.2%

of total). They consisted in the $\alpha 1H$ subunit of the voltage-dependent T type calcium channel (*CACNA1H*), the large subunit of the calpain 1 (*CAPN1*), the dystrophin (*DMD*), the NMDA ionotropic glutamate receptor 2B (*GRIN2B*), the junctophilin 2 (*JPH2*), the mannosidase α class 1A (*MAN1A1*), the X-linked protocadherin 11 (*PCDH11X*), the protein interacting with cyclin A1 (*PROCA1*), the slit homolog 2 (*SLIT2*), the erythroid $\alpha 1$ spectrin (*SPTA1*), and the tescalcin (*TESC*) (**Table 3**).

An unsupervised clustering including all the somatic mutations in genes involved in the cAMP pathway or in the Ca^{2+} signalling was performed. The results, including the relationship with the total number of somatic mutations and clinical data is shown in the **Figure 2**.

Concerning the correlation with the clinical data, the patients with mutations in genes of the cAMP signalling pathway were mostly females (10/14, 71%), while those with mutations in genes associated with the Ca^{2+} signalling were mostly males (5/7, 71%) and those with other kinds of mutations were equally distributed between the two sexes (50%, $P < 0.001$ by Kruskal-Wallis test for multiple comparisons) (**Figure 2** and **Figure 3**). A trend to a lower total number of mutations and younger age was observed in the group of patients without alterations of the cAMP or Ca^{2+} signalling in comparison with the other two groups (**Figure 2** and **Figure 3**). No significant differences in terms of tumor size and basal GH or IGF1 levels have been found.

Functional annotation and pathway analysis: The gene enrichment analysis in the entire series identified a total of 117 altered genes associated with a gene ontology (GO) term. Several altered genes resulted to be associated with developmental biological processes (**Supplementary table 4**). The canonical pathway analysis recognized no significant overlaps. The gene family analysis showed the presence of 1 cytokine/growth factor (*SLIT2*), 7 protein kinases (*ADRBK2*, *CDK10*, *CHUK*, *EPHA8*, *PRKAA2*, *SCYLI*, *TESK1*), 4 known oncogenes (*GNAS*, *KDM5A*, *SH3GL1*, *STIL*) and 2 tumor suppressor genes (*SETD2*, *TSC2*) among the mutated genes.

Discussion

The present study offers a comprehensive genetic characterization of a large cohort of 67 GH-secreting pituitary adenomas. We aimed to identify novel molecular markers potentially involved in

tumorigenesis and/or in clinical outcome. To this end, we first performed targeted sequencing of *GNAS*, *PRKACA* and *USP8* genes in order to evaluate the presence of mutations in these genes in GH-secreting adenomas, finding only known *GNAS* gene mutations. By whole-exome sequencing, only a limited number of genetic alterations have been detected in the 36 evaluated samples. This finding is consistent with the low mitotic activity of pituitary tumors and with previous small studies on both non-functioning (n=7)⁴³ and GH-secreting pituitary adenomas (n=12)³⁷. Moreover, no recurrent somatic mutations have been observed, except the known alterations at the *GNAS* gene, similarly to a previous report on a small series of GH-secreting adenomas³⁷. In particular, no somatic mutations have been also detected at the gene *GPR101*, probably due to the low reported frequency of this mutations (11/248 cases)³³, and, at both the exome-sequencing and the targeted sequencing, we did not find any mutations of the *PRKACA* and *USP8* genes. These findings further confirm that both these genetic alterations are not involved in the pathogenesis of GH-secreting adenomas^{32, 36}.

Interestingly, several non-recurrent alterations affected other genes involved in the cAMP signalling besides *GNAS* (see Table 2). These findings further support the view that deregulation of cAMP pathway is the most important pathogenetic mechanism in GH-secreting adenomas. Furthermore, a number of genes associated with the Ca^{2+} signalling (see Table 2) were altered. These findings are in agreement with another recent study on whole-genome alterations in 12 GH-secreting adenomas³⁷. This is consistent with the notion that binding of GHRH to its receptor activates not only the stimulatory subunit α of the G-protein ($\text{G}\alpha\text{-S}$, cAMP-dependent pathways), but also $\text{G}\alpha\text{-I}$, $\text{G}\beta$ and $\text{G}\gamma$ leading to release of intracellular free Ca^{2+} , which then further triggers secretion of GH^{44, 45}. Moreover, ATP, which is co-released with pituitary hormones, induces an increase in free Ca^{2+} in pituitary cells⁴⁶. These data strongly suggest that dysregulation of the calcium signalling might be an important co-signal in somatotrophs and potentially involved in pituitary tumorigenesis. **However, its biological role needs to be better investigated in future functional studies.**

It has been suggested that tumors might be very heterogeneous with few mutations in common. Instead, different genes acting through the same molecular pathways may contribute to tumor formation⁴⁷. Therefore, it is possible that at least some of these low-frequency GH-secreting tumor variants present tumor promoting mutations. Alternatively, they may present other types of

molecular alterations not detectable by exome-sequencing (i.e. mutations in non-coding intronic chromosomal regions).

In conclusion, we found no novel recurrently mutated genes in a large series of GH-secreting pituitary adenomas. However, our and previous genetic findings suggest that beside cAMP pathway, also different pathways, such as Ca^{2+} signalling, may play an important role in the pathogenesis of these tumors.

Declaration of interest

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution

C.L. Ronchi, B. Allolio, M. Reincke und M. Fassnacht conceived the idea of the study. C.L. Ronchi developed the protocol of the study, coordinated the collection of the tissue materials and the clinical data, performed the statistical analysis and wrote the first draft of the paper; E. Peverelli, G. Mantovani, A. Spada, provided the tumor tissue and the corresponding blood samples and contributed to wrote the paper; S. Herterich performed the targeted sequencing analysis; I. Weigand, D. Calebiro and S. Sbiera contributed to the protocol of the study and to the data analysis; S. Appenzeller performed the clustering and the heatmap; J. Honegger, M. Reincke, M. Buchfelder, and J. Flitsch provided the tumor tissue and the corresponding blood samples; T. Strom performed the whole-exome sequencing analysis including the filtering and the first data analysis; M. Fassnacht contributed to the coordination of the study and to write the paper. All the authors reviewed and approved the final version of the manuscript.

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References

1. Chanson P, Salenave S, Kamenicky P, Cazabat L & Young J. Pituitary tumours: acromegaly. *Best Pract Res Clin Endocrinol Metab* 2009 **23** 555-574.
2. Sherlock M, Ayuk J, Tomlinson JW, Toogood AA, Aragon-Alonso A, Sheppard MC, Bates AS & Stewart PM. Mortality in patients with pituitary disease. *Endocr Rev* 2010 **31** 301-342.
3. Melmed S, Kleinberg DL, Bonert V & Fleseriu M. Acromegaly: assessing the disorder and navigating therapeutic options for treatment. *Endocr Pract* 2014 **20 Suppl 1** 7-17; quiz 18-20.
4. Asa SL & Ezzat S. The pathogenesis of pituitary tumours. *Nat Rev Cancer* 2002 **2** 836-849.
5. Clayton RN & Farrell WE. Pituitary tumour clonality revisited. *Front Horm Res* 2004 **32** 186-204.
6. Vallar L, Spada A & Giannattasio G. Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. *Nature* 1987 **330** 566-568.
7. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR & Vallar L. GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* 1989 **340** 692-696.
8. Landis CA, Harsh G, Lyons J, Davis RL, McCormick F & Bourne HR. Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant Gs protein. *J Clin Endocrinol Metab* 1990 **71** 1416-1420.
9. Clementi E, Margaretti N, Meldolesi J & Taramelli R. A new constitutively activating mutation of the Gs protein alpha subunit-gsp oncogene is found in human pituitary tumours. *Oncogene* 1990 **5** 1059-1061.
10. Lania AG, Mantovani G, Ferrero S, Pellegrini C, Bondioni S, Peverelli E, Braidotti P, Locatelli M, Zavanone ML, Ferrante E, et al. Proliferation of transformed somatotroph cells related to low or absent expression of protein kinase a regulatory subunit 1A protein. *Cancer Res* 2004 **64** 9193-9198.
11. Vitali E, Peverelli E, Giardino E, Locatelli M, Lasio GB, Beck-Peccoz P, Spada A, Lania AG & Mantovani G. Cyclic adenosine 3'-5'-monophosphate (cAMP) exerts proliferative and anti-proliferative effects in pituitary cells of different types by activating both cAMP-dependent protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac). *Mol Cell Endocrinol* 2014 **383** 193-202.
12. Hayward BE, Barlier A, Korbonits M, Grossman AB, Jacquet P, Enjalbert A & Bonthron DT. Imprinting of the G(s)alpha gene GNAS1 in the pathogenesis of acromegaly. *J Clin Invest* 2001 **107** R31-36.
13. Mantovani G, Ballare E, Giammona E, Beck-Peccoz P & Spada A. The gsalph gene: predominant maternal origin of transcription in human thyroid gland and gonads. *J Clin Endocrinol Metab* 2002 **87** 4736-4740.
14. Mantovani G, Bondioni S, Lania AG, Corbetta S, de Sanctis L, Cappa M, Di Battista E, Chanson P, Beck-Peccoz P & Spada A. Parental origin of Gsalpha mutations in the McCune-Albright syndrome and in isolated endocrine tumors. *J Clin Endocrinol Metab* 2004 **89** 3007-3009.
15. Picard C, Silvy M, Gerard C, Buffat C, Lavaque E, Figarella-Branger D, Dufour H, Gabert J, Beckers A, Brue T, et al. Gs alpha overexpression and loss of Gs alpha

- imprinting in human somatotroph adenomas: association with tumor size and response to pharmacologic treatment. *Int J Cancer* 2007 **121** 1245-1252.
16. Petersenn S, Heyens M, Ludecke DK, Beil FU & Schulte HM. Absence of somatostatin receptor type 2 A mutations and gip oncogene in pituitary somatotroph adenomas. *Clin Endocrinol (Oxf)* 2000 **52** 35-42.
 17. Dong Q, Brucker-Davis F, Weintraub BD, Smallridge RC, Carr FE, Battey J, Spiegel AM & Shenker A. Screening of candidate oncogenes in human thyrotroph tumors: absence of activating mutations of the G alpha q, G alpha 11, G alpha s, or thyrotropin-releasing hormone receptor genes. *J Clin Endocrinol Metab* 1996 **81** 1134-1140.
 18. Farrell WE, Talbot JA, Bicknell EJ, Simpson D & Clayton RN. Genomic sequence analysis of a key residue (Arg183) in human G alpha q in invasive non-functional pituitary adenomas. *Clin Endocrinol (Oxf)* 1997 **47** 241-244.
 19. Pack SD, Kirschner LS, Pak E, Zhuang Z, Carney JA & Stratakis CA. Genetic and histologic studies of somatomammotropic pituitary tumors in patients with the "complex of spotty skin pigmentation, myxomas, endocrine overactivity and schwannomas" (Carney complex). *J Clin Endocrinol Metab* 2000 **85** 3860-3865.
 20. Kirschner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C, Cho YS, Cho-Chung YS & Stratakis CA. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. *Nat Genet* 2000 **26** 89-92.
 21. Yamasaki H, Mizusawa N, Nagahiro S, Yamada S, Sano T, Itakura M & Yoshimoto K. GH-secreting pituitary adenomas infrequently contain inactivating mutations of PRKAR1A and LOH of 17q23-24. *Clin Endocrinol (Oxf)* 2003 **58** 464-470.
 22. Sandrini F, Kirschner LS, Bei T, Farmakidis C, Yasufuku-Takano J, Takano K, Prezant TR, Marx SJ, Farrell WE, Clayton RN, et al. PRKAR1A, one of the Carney complex genes, and its locus (17q22-24) are rarely altered in pituitary tumours outside the Carney complex. *J Med Genet* 2002 **39** e78.
 23. Horvath A, Mathyakina L, Vong Q, Baxendale V, Pang AL, Chan WY & Stratakis CA. Serial analysis of gene expression in adrenocortical hyperplasia caused by a germline PRKAR1A mutation. *J Clin Endocrinol Metab* 2006 **91** 584-596.
 24. Horvath A, Boikos S, Giatzakis C, Robinson-White A, Groussin L, Griffin KJ, Stein E, Levine E, Delimpasi G, Hsiao HP, et al. A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. *Nat Genet* 2006 **38** 794-800.
 25. Peverelli E, Ermetici F, Filopanti M, Elli FM, Ronchi CL, Mantovani G, Ferrero S, Bosari S, Beck-Peccoz P, Lania A, et al. Analysis of genetic variants of phosphodiesterase 11A in acromegalic patients. *Eur J Endocrinol* 2009 **161** 687-694.
 26. Beuschlein F, Fassnacht M, Assie G, Calebiro D, Stratakis CA, Osswald A, Ronchi CL, Wieland T, Sbiera S, Faucz FR, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. *N Engl J Med* 2014 **370** 1019-1028.
 27. Cao Y, He M, Gao Z, Peng Y, Li Y, Li L, Zhou W, Li X, Zhong X, Lei Y, et al. Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science* 2014 **344** 913-917.
 28. Sato Y, Maekawa S, Ishii R, Sanada M, Morikawa T, Shiraishi Y, Yoshida K, Nagata Y, Sato-Otsubo A, Yoshizato T, et al. Recurrent somatic mutations underlie corticotropin-independent Cushing's syndrome. *Science* 2014 **344** 917-920.
 29. Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, Kunstman JW, Korah R, Suttorp AC, Dietrich D, et al. Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nat Genet* 2014 **46** 613-617.
 30. Di Dalmazi G, Kisker C, Calebiro D, Mannelli M, Canu L, Arnaldi G, Quinkler M, Rayes N, Tabarin A, Laure Jullie M, et al. Novel somatic mutations in the catalytic

- subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study. *J Clin Endocrinol Metab* 2014 **99** E2093-2100.
31. Calebiro D, Hannawacker A, Lyga S, Bathon K, Zabel U, Ronchi C, Beuschlein F, Reincke M, Lorenz K, Allolio B, et al. PKA catalytic subunit mutations in adrenocortical Cushing's adenoma impair association with the regulatory subunit. *Nat Commun* 2014 **5** 5680.
 32. Larkin SJ, Ferrau F, Karavitaki N, Hernandez-Ramirez LC, Ansorge O, Grossman AB & Korbonits M. Sequence analysis of the catalytic subunit of PKA in somatotroph adenomas. *Eur J Endocrinol* 2014 **171** 705-710.
 33. Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, Schernthaner-Reiter MH, Szarek E, Leal LF, et al. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *N Engl J Med* 2014 **371** 2363-2374.
 34. LeRiche VK, Asa SL & Ezzat S. Epidermal growth factor and its receptor (EGF-R) in human pituitary adenomas: EGF-R correlates with tumor aggressiveness. *J Clin Endocrinol Metab* 1996 **81** 656-662.
 35. Chaidarun SS, Eggo MC, Sheppard MC & Stewart PM. Expression of epidermal growth factor (EGF), its receptor, and related oncoprotein (erbB-2) in human pituitary tumors and response to EGF in vitro. *Endocrinology* 1994 **135** 2012-2021.
 36. Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, Meitinger T, Mizuno-Yamasaki E, Kawaguchi K, Saeki Y, et al. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nat Genet* 2015 **47** 31-38.
 37. Valimaki N, Demir H, Pitkanen E, Kaasinen E, Karppinen A, Kivipelto L, Schalin-Jantti C, Aaltonen LA & Karhu A. Whole-Genome Sequencing of Growth Hormone (GH) - secreting Pituitary Adenomas. *J Clin Endocrinol Metab* 2015 jc20153129.
 38. Ronchi CL, Leich E, Sbiera S, Weismann D, Rosenwald A, Allolio B & Fassnacht M. Single nucleotide polymorphism microarray analysis in cortisol-secreting adrenocortical adenomas identifies new candidate genes and pathways. *Neoplasia* 2012 **14** 206-218.
 39. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R & Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* 2007 **35** W71-74.
 40. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS & Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods* 2010 **7** 248-249.
 41. Kumar P, Henikoff S & Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009 **4** 1073-1081.
 42. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005 **102** 15545-15550.
 43. Newey PJ, Nesbit MA, Rimmer AJ, Head RA, Gorvin CM, Attar M, Gregory L, Wass JA, Buck D, Karavitaki N, et al. Whole-exome sequencing studies of nonfunctioning pituitary adenomas. *J Clin Endocrinol Metab* 2013 **98** E796-800.
 44. Meier K, Knepel W & Schofl C. Potassium depolarization elevates cytosolic free calcium concentration in rat anterior pituitary cells through 1,4-dihydropyridine-sensitive, omega-conotoxin-insensitive calcium channels. *Endocrinology* 1988 **122** 2764-2770.
 45. Canny BJ, Rawlings SR & Leong DA. Pituitary adenylate cyclase-activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes. *Endocrinology* 1992 **130** 211-215.

46. Villalobos C, Alonso-Torre SR, Nunez L & Garcia-Sancho J. Functional ATP receptors in rat anterior pituitary cells. *Am J Physiol* 1997 **273** C1963-1971.
47. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, et al. The genomic landscapes of human breast and colorectal cancers. *Science* 2007 **318** 1108-1113.

Legend to the figures

Figure 1. Total number of somatic mutations in the 36 GH-secreting pituitary tumors evaluated by next-generation exome-sequencing (Group 2). The tumors affected by mutations in *GNAS* are represented with red bars. The numeration of the GH secreting adenomas is consecutive and do not correspond to the tumor identification number.

Figure 2. Overview of the somatic mutations at genes involved in the cAMP signalling (i.e. *CCR10*, *OR51B4*, *CHRM3*, *GNAS*, *PRKAA2*, *GRK3*, *ATP6V0A1*) or in the calcium signalling (i.e. *CACNA1H*, *CAPN1*, *DMD*, *GRIN2B*, *JPH2*, *MAN1A1*, *PCDH11X*, *PROCA1*, *SLIT2*, *SPTA1*, *TESC*) in GH-secreting adenomas evaluated by next-generation exome-sequencing (n=36) and relationship with the total number of somatic mutations, sex, basal GH levels, and tumor extension at the time of diagnosis. Age: child < 18 years, young ≤50 years (median), old >50 years; tumor size: macro=macroadenoma, micro=microadenoma; tumor extension: extra=extrasellar, intra=intracellular; GH: low ≤15 µg/l (median), high >15 µg/l.

Figure 3. Relationship between the genetic alterations observed at the exome-sequencing (i.e. mutations in genes member of the cAMP pathway, of the calcium signalling or in others) and clinical data (i.e. total number of somatic mutations in upper panel, age in the middle panel; sex in the lower panel) in 36 evaluated GH-secreting adenomas.

Suppl Table 3. List of all genetic alterations observed in 36 GH-secreting pituitary tumors by next-generation exome-sequencing (Group 2), including gene symbols, transcripts and aminoacid substitutions.